PATENT COOPERATION TREATY

09/786435

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Perrey, Ralf
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Eingegangen
02. JAN. 2001
Müller-Boré & Partner
Frist:

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	29.12.2000
Applicant's or agent's file reference: B 1957 - py	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/06433	01/09/1999	03/09/1998

Applicant: BIOPHARM GESELLSCHAFT ZUR BIOTEC... et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Danti, B Tel.+49 89 2399-8161

Form PCT/IPEA/416 (July 1992)

ATENT COOPERATION TREATY 09/786435

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
B 1957 - py		

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/06433	01/09/1999	03/09/1998

International Patent Classification (IPC) or national classification and IPC
A61K39/395

Applicant
BIOPHARM GESELLSCHAFT ZUR BIOTEC... et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 10 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
31/03/2000	29.12.2000

Name and mailing address of the international preliminary examining authority:
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Authorized officer
Giry, M
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Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/06433

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-4	as originally filed			
5-12	as received on	07/12/2000	with letter of	07/12/2000

Claims, No.:

1-8	as received on	07/12/2000	with letter of	07/12/2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5-8
	No:	Claims	1-4
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-8
Industrial applicability (IA)	Yes:	Claims	
	No:	Claims	

2. Citations and explanations
   s e separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 - **Reference** is made to the following documents :

D1 : WO 95 10611 A, 20 April 1995
D2 : WO 93 19783 A, 14 October 1993
D3 : EP-A-0 181 267, 14 May 1986

2 - **Novelty** - Art. 33(1) and (2) PCT :

2.1 Document D1 discloses a method for inducing neuronal differentiation and preventing the death or degeneration of neuronal cells *in vivo* by antagonizing a signalling pathway for a growth factor of the TGF-β family and pharmaceutical preparations comprising a neutralizing agent capable of antagonizing said pathway (Abstract ; p. 18, lines 7-11). This method is applied therapeutically and can be used to treat neurodegenerative disorders associated with the progressive loss of neuronal cells such as neuronal damage resulting from anoxia-ischemia (p. 5, lines 4-11 ; p. 45-48, claims 1, 16, 27-40). The method comprises the use of an agent capable of antagonizing the biological action of a protein of the TGF-β family, the antagonist acting by competitive or non-competitive binding to a cell-surface receptor for the growth factor, sequestration of the growth factor or inhibition of signal transduction events mediated by the growth factor receptor (p. 5, line 33 to p. 6, line 4). The applicant's representative argues that document D1 relates to the differentiation of neural progenitor cells, *i.e.* cells that are totally different from those of the present application, and that D1 can therefore not be taken into consideration. This argument cannot be accepted since the present invention concerns compounds capable of inhibiting the biological activity of TGF-β on damaged neurons due to ischemia or a neurodegenerative disorder (claims 1-4 ; p. 2, lines 5-7) without further limiting said compounds mode of action to a certain neural cell type. Since the method described in D1 is explicitly "amenable to treat neurodegenerative disorders associated with neuronal damage resulting

from anoxia-ischemia and the loss of neuronal cells" (p. 5, lines 4-11), document D1 anticipates the subject-matter of claims 1-4.

2.2 Document D2 describes methods for treating central nervous system (CNS) pathologies by contacting the tissue with an agent able to inhibit the biological activity of TGF-β1, for example a neutralizing anti TGF-β1 antibody or agents that act as TGF-β antagonists, such as fragments of TGF-β able to specifically bind to a TGF-β receptor, and competitively prevent the TGF-β binding to its receptor (p. 8, line 20 to p. 9, line 2). Pharmaceutical compositions containing said agents are also mentioned (p. 10, line 10-14). The use of TGF-β1 antagonists in therapies designed to promote regeneration of damaged neural pathways is explicitly envisaged (p. 8, lines 12-19). Consequently, for the same reasons as those given under point 2.1, document D2 also appears to be novelty destroying for claims 1-4.

2.3 Since none of the available prior art documents mention any pharmaceutical composition containing a first compound able to inhibit the biological activity of TGF-β and a second compound for disintegrating blood clots, the subject-matter of claims 5-8 can be seen as novel.

3 - **Inventive step** - Art. 33(1) and (3) PCT :

3.1 The closest prior art document D1 mentions pharmaceutical compositions comprising neutralizing agents acting as antagonists directed against the biological activity of a protein of the TGF-β family (see above point 2.1). The subject-matter of claim 5 concerning a pharmaceutical composition differs from D1 by the presence of a second compound capable of disintegrating blood clots. The problem to be solved by the present application can therefore be seen in providing an alternative pharmaceutical composition for treating cerebral disorders.

3.2 Document D1 mentions that "the neutralizing agent can be administered as part of a combinatorial therapy with other agents" (p. 19, lines 23-24). Compounds containing a truncated receptor for a growth factor of the TGF-β family, *i.e.*

compounds having the binding site of a TGF-β receptor, are given as examples of neutralizing agents (p. 4, lines 25-33). The subject-matter of claims 5-7 could only be regarded as inventive, if the composition to which they relate presents unexpected effects or properties. However, no such effects or properties are indicated in the application (see also point VIII). Hence, no inventive step is present in the subject-matter of claims 5-7.

3.3 Tissue plasminogen activator (tPA) and urokinase (UK), referred to in claim 8, have already been disclosed in document D3 as plasminogen activators acting as thrombolytic agents implicated in the dissolution of thrombi *i.e.* blood clots (p. 1, lines 10-21). Therefore, document D3 not only belongs to the same technical field as that of the invention, but also, since these compounds are described in document D3 as providing the same advantages as in the present application, the skilled person would therefore regard it as a normal option to include them in pharmaceutical compositions for treating cerebral disorders, especially as the advantages thus achieved can readily be foreseen. Consequently, the subject-matter of claim 8 cannot be considered as inventive.

4 - **Industrial applicability** - Art. 33(1) and (4) PCT :

For the assessment of the present claims 1-8, relating to the use of a compound for the preparation of a medicament for treating cerebral disorders and a pharmaceutical composition for use in said medical treatment, on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

The application does not provides any example of a pharmaceutical composition as claimed in claims 5-8 or of its use. The description only refers to the fact that "adverse consequences of CNS injuries may be caused by thrombus ..." (p. 2, line 33 to p. 3, line 30), which is a mere hypothesis with no support whatsoever (Art. 5 and 6 PCT).

Application No.: [redacted]/EP99/06433
Applicant: Biopharm Gesellschaft zur biotechnologischen Entwicklung und zum Vertrieb von Pharmaka mbH
"Use of TGF-Inhibitors for treating cerebral disorders"
Our Ref: B 1957 - py / js

New pages 5 to 12 of the description

The following example demonstrates the specifity of anti-TGF-β-antibody as well as morphology and neuron numbers of chick ganglia at E10, i.e. after the main period of ontogenetic ciliary neuron death. Embryos were treated daily from E6 to E9 with 10 μg of a monoclonal mouse anti-TGF-β1, -β2, -β3 antibody (anti-TGF-β; obtained from Genzyme) applied to the chorionic allantoic membrane, an identical dose of a mouse IgG with or without rhodamine conjugation, or 2 μg of a recombinant human TGF-β RII/Fc chimeric protein (TβR-II-Fc; obtained form R&D Systems). Ciliary ganglia were dissected at E10, fixed in Bouin's solution, and paraffin-embedded. Dot blots were prepared showing the specifity of the anti-TGF-β-antibody. Furthermore, sections (H.E. stain or fluorescence microscopy) through the largest circumference of ganglia from embryos were prepared and neuron counts in ganglia with the treatments according to the present invention displayed increased neuron numbers as compared to untreated embryos.

Furthermore, neuron numbers and apoptosis in ciliary ganglia (CG), dorsal root ganglia (L3; DRG), and the lumbar motoneuron column of embryos treated with anti-TGF-β were examined. Neuron counts at E10 following daily treatments from E6 - E9 and TUNEL labelings of CG, DRG, and lumbar motoneurons at E8 following treatment with anti-TGF-β-antibody at E6 and E7 were compared to untreated embryos. A quantitative analysis of TUNEL-positive neurons demonstrated increased or decreased neuron numbers as compared to untreated embryos.

Neuron counts showed a phenotype rescue of CG and motoneurons in anti-TGF-ß-treated embryos by administration of TGF-ß. A treatment with TGF-ß3

(2μg) at E10 following administration of anti-TGF-ß from E6 to E9 produced neuron numbers at E14 identical to control embryos. Continuing treatment with anti-TGF-ß from E10 to E 13 fully maintained neuron numbers.

Qualitative microscopic and quantitative analyses of H.E. stained sections showed that anti-TGF-ß rescues lumbar DRG and motoneurons following unilateral limb bud ablation.

The present invention is illustrated by the following non-limiting example.

EXAMPLE

Neuron counting experiments

Chick embryos were treated daily with 50 μl (phosphate buffered saline, supplemented with 200 units/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml neomycin (each obtained from Gibco) containing either 10 μg of a pan anti-TGF-β (obtained from Genzyme) or 2 μg TβR-II-Fc (obtained from R&D Systems), or 10 μg mouse IgG (obtained from Sigma) by administration onto the chorioallantoic membrane through a window in the shell as described in Oppenheim et al., 1993. Embryos were killed on either E8, E10 or E14. The CG and the lumbar spinal cord with attached DRG were dissected, fixed in Bouin's solution and paraffin embedded. All tissues were sectioned at 8 to 10 μm and stained with H.E. Neurons were counted in every fifth or tenth section as described in Oppenheim et al., 1993.

TUNEL labeling

Sections were stained according to the manufacturer's instructions (Boehringer Mannheim) and counter-stained with H.E. TUNEL positive nuclei of large neurons were counted on every tenth section and expressed as ratio of total neuron of this particular neuron population.

Unilateral limb bud ablation

Ablations of hind limb buds of chick embryos were performed at E3. Embryos were treated daily (10 µg; E3 - E6) as indicated above with either anti-TGF-ß or IgG and processed at E7 for H.E. staining and neuron counting.

Anti-TGF-β-antibody neutralizes endogenous TGF-β during the main period of ontogenetic neuron cell death

In the developing nervous system of chick and mammals, TGF-ß2 and -ß3 as well as their receptors occur in many populations of postmitotic neurons, including CG, DRG, and motoneurons (Krieglstein et al., 1998). A monoclonal antibody recognizing all three isoforms of TGF-ß (anti-TGF-ß) was used to neutralize endogenous TGF-ß during the main period of ontogenetic cell death (E6 - E9) of CG and DRG as well as spinal motoneurons. Ten µg of this antibody neutralize 10 ng of each available recombinant TGF-ß including chicken TGF-ß3 (R&D Systems) to >98%, as assessed in a bioassay using mink lung epithelial cells (Abe et al., 1994; Krieglstein and Unsicker, 1995). The specifity of this monoclonal anti-TGF-ß antibody was ascertained by dot blot using 40 other growth factors. Daily treatments with anti-TGF-ß resulted in an increased size of CG at E10. A corresponding control IgG, which is rhodamine-conjugated, could be detected throughout the entire embryo. A TßR-II-Fc chimeric protein employed as an alternative tool to capture endogenous TGF-ß likewise increased the size of CG at E10 compared to untreated or IgG-treated embryos. Neuron counts revealed that the increased size of CG is due to a significant increase in neuron numbers. The counted values of approximately 6,000 reflect neuron numbers prior to ontogenetic neuron death at E6 (Landmesser and Pilar, 1974) showing that elimination of TGF-ß signalling prevents the execution of ontogenetic neuron death in the chick CG.

Neutralizing TGF-ß interferes with ontogenetic cell death in sensory and motoneurons

Sensory and motoneurons were analyzed to investigate whether neutralizing TGF-ß also interfered with the development of other neuron populations during the period of ontogenetic neuron death. Neuron counts in the L3 DRG and in the lumbar motoneuron column revealed a significant increase in neuron numbers at E10 following daily antibody treatments from E6 to E9. Numbers of motoneurons in antibody-treated embryos almost matched neuron numbers prior to the onset of ontogenetic neuron death (Hamburger et al., 1975). To determine whether the increase in numbers of neurons upon anti-TGF-ß-treatment resulted from reduced ontogenetic neuron death, TUNEL labelling was performed to stain for apoptotic cells. Counting the numbers of TUNEL-positive cells at E8, which is the peak of cell death for all three neuron populations investigated, revealed a significant reduction of the proportion of apoptotic cells. This shows that endogenous TGF-ß is essential for executing the cell death program in developing neurons.

Ontogenetic neuron death occurs after termination of antibody treatment and substitution of endogenous TGF-β

Furthermore, it was investigated whether ontogenetic neuron death would occur at a later time point following termination of the antibody treatment and substitution of endogenous TGF-β. A single dose of TGF-β3 at E10 after the end of the antibody treatment (E6 -E9) resulted in a significant reduction of neuron numbers in the CG and motoneuron column. Numbers of neurons matched those of control embryos at the end of the main ontogenetic cell death period (E10) as well as embryos at E14. Therefore, TGF-β mediates death of selected neurons, especially those that are destined to die. In contrast, prolongation of the antibody treatment beyond the period of ontogenetic death stabilized neuron numbers at increased levels.

Treatment of limb bud-ablated embryos with anti-TGF-β rescues both motoneurons and DRG neurons

Multiple evidence suggests that the extent of ontogenetic neuron death is crucially regulated by the target (Olek and Edwards, 1978; Pittman and Oppenheim, 1979; Pilar et al., 1980; Thoenen and Edgar, 1985), best exemplified by the classic experiments of V. Hamburger (Hollyday and Hamburger, 1976). Extirpation of the hind limb bud in the E3 chick embryo, i.e. prior to the arrival and synapse formation of motoneuron axons in the target (Dahm and Landmesser, 1988, 1991), causes death of almost all lumbar motoneurons and sensory neurons by E7 (Oppenheim et al., 1978). Qualitative microscopic analysis documented that treatment of limb bud-ablated embryos with anti-TGF-ß rescues both motoneurons as well as DRG neurons. Quantification reveals that the lumbar motoneuron population in limb-ablated embryos could surprisingly be rescued by anti-TGF-ß treatment to approximately half the half the size as compared to the non-operated control side.

Summary

The above results show that TGF-ß exerts a key role both in the regulation of ontogenetic neuron death of three major classes of peripheral and CNS neurons as well as in the regulation of cell death following target ablation. Parasympathetic CG, sensory DRG, and spinal lumbar motoneurons in chick embroys have their main period of ontogenetic neuron death between E6 and E9, loose approximately 50% of the total cells generated, but require largely distinct and only partly overlapping target-derived trophic molecules to secure the survival of optimal cell numbers. CG neurons are maintained by CNTF/GPA, DRG neurons mainly by neurotrophins, and motoneurons by GDNF, HGF, neurotrophins, IGF-I and others (Nishi, 1994; Heller et al., 1995; Lewin and Barde, 1996; Oppenheim, 1996). In motoneurons, even a cocktail of numerous trophic molecules cannot fully maintain the population. The example of the present invention demonstrates that the elimination of endogenous TGF-β or TGF-β signaling, respectively, results in rescuing effects, which are distinct from neurotrophic

factor treatments in terms of (i) the broad spectrum of responsive populations and (ii) magnitude of effect, which encompasses virtually all neurons of a given population. Therefore, the lack of TGF-β is able to rescue all neurons destined to die. The elimination of TGF-β is superior to any of known manipulations of the molecular cascade involved in neuron cell death in that neuron populations are fully maintained.

The example shows that ontogenetic neuron death of ciliary, dorsal root and spinal motoneurons is largely prevented and neuron losses following limb bud ablation are greatly reduced following neutralization of endogenous TGF-β by an anti-TGF-β antibody in chick embryos. Likewise, preventing TGF-β signaling by treatment with a TβR-II fusion protein during the period of ontogenetic cell death in the ciliary ganglion rescues all neurons, which normally die. TUNEL staining revealed decreased numbers of apoptotic cells. Application of exogenous TGF-β rescued the deprived phenotype. Thus, TGF-β in contrast to any single neurotrophic factor acting in the above systems plays a key role in regulating ontogenetic neuron death as well as cell death following neuronal target deprivation.

The above results demonstrate the pivotal role of TGF-β in regulating neuron survival and death. The data given above show that TGF-β is an essential trigger of neuronal death and thus, compounds according to the present invention which are capable of inhibiting the biological activity of TGF-β as e.g. TGF-β-specific antibodies are exceptionally good therapeutic tools that prevent TGF-β signalling in the treatment of stroke, neurotrauma, and neurodegenerative diseases.

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Application No.: PCT/EP99/06433
Applicant: Biopharm Gesellschaft zur biotechnologischen Entwicklung und zum Vertrieb von Pharmaka mbH
"Use of TGF-Inhibitors for treating cerebral disorders"
Our Ref: B 1957 - py / js

Claims

1. Use of a compound capable of inhibiting the biological activity of TGF-β on predamaged neurons, for the preparation of a medicament for treating cerebral disorders.

2. Use according to claim 1, wherein said compound is an antibody or an antagonist directed to TGF-β.

3. Use according to claim 1 or 2, wherein said disorder is a peripheral and/or CNS-disorder.

4. Use according to claim 3, wherein said disorder is a cerebral ischemia or a neurodegenerative disorder.

5. A pharmaceutical composition containing, in pharmaceutically effective amounts, a compound capable of inhibiting the biological activity of TGF-β on predamaged neurons, and a second compound for disintegrating blood clots.

6. The pharmaceutical composition according to claim 5 further containing a pharmaceutically acceptable carrier and/or diluent.

7. The pharmaceutical composition according to claim 5 or 6, wherein said compound is an antibody directed to TGF-β or a compound having the binding site of a TGF-β receptor.

8. The pharmaceutical composition according to anyone of claims 5 to 7, wherein said second compound is selected from the group consisting of urokinase and tissue plasminogen activator.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 May 2000 (02.05.00)	
International application No. PCT/EP99/06433	**Applicant's or agent's file reference** B 1957 - py
International filing date (day/month/year) 01 September 1999 (01.09.99)	**Priority date** (day/month/year) 03 September 1998 (03.09.98)
Applicant KRIEGELSTEIN, Kerstin	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

31 March 2000 (31.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Claudio Borton
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
B 1957 - py		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/ 06433	01/09/1999	03/09/1998
Applicant		
BIOPHARM GESELLSCHAFT ZUR BIOTEC... et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   USE OF TGF-BETA INHIBITORS FOR TREATING CEREBRAL DISORDERS

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. --

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/06433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61K38/48 A61K38/49 //(A61K39/395,38:48), (A61K39/395,38:49)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 10611 A (HARVARD COLLEGE) 20 April 1995 (1995-04-20) page 15, line 29 -page 17, line 35 claims 1-5,13,22,27,35,36 ---	1-4
X	WO 93 19783 A (WHITTIER INSTITUTE FOR DIABETES AND ENDOCRINOLOGY) 14 October 1993 (1993-10-14) the whole document --- -/--	1-4

[X] Further documents are listed in the continuation of box C.

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25 November 1999	09/12/1999

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/EP 99/06433

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(54) Title: USE OF TGF–BETA INHIBITORS FOR TREATING CEREBRAL DISORDERS

(57) Abstract

The present invention relates to the use of a compound capable of substantially inhibiting the biological activity of TGF–β on predamaged neurons, for treating cerebral disorders, and to pharmaceutical compositions containing said compound and a second compound for disintegrating blood clots.

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USE OF TGF-BETA INHIBITORS FOR TREATING CEREBRAL DISORDERS

Description

The present invention relates to the use of a compound capable of substantially inhibiting the biological activity of TGF-β on predamaged neurons, for treating cerebral disorders, and to pharmaceutical compositions containing said compound and a second compound for disintegrating blood clots.

The transforming growth factor β (TGF-β) family contains subspecies TGF-β1, TGF-β2 and TGF-β3, which are widely distributed and contextually acting cytokines with prominent roles in development and cell cycle control (Roberts and Sporn, 1990; Kriegelstein et al., 1995). TGF-βs have been implicated in the regulation of neuronal survival of e.g. motoneurons, sensory and midbrain dopaminergic neurons. The TGF-β isoforms show widely overlapping expression patterns. Nullmutations for each of these isoforms show distinct phenotypes, which, however, are restricted to non-neural tissues (Shull et al., 1992; Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997). Mice deficient for the TGF-ß receptor type-II (TβR-II) are lethal at E 10.5, i.e. prior to the development of the nervous system.

During the development of the vertebrate nervous system, large numbers of neurons in the central and peripheral nervous system undergo naturally occuring cell death (Oppenheim, 1991). Regulation of neuron survival or death, respectively, requires the concerted actions of sets of molecules, which act cell-extrinsically and -instrinsically to execute or prevent cell death (Pettmann and Henderson, 1998).

Cerebral disorders such as a neurodegenerative disorder or cerebral ischemiae, result in injury or death of neurons in mammals, and produce motor and/or

cognitive deficits that are often permanent. At present, in most of these cerebral disorders there is no treatment that reliably improves the prognosis of a patient suffering from said disorders.

Thus, the technical problem underlying the present invention is to provide a new system imparting protection and therefore survival on predamaged or injured neurons upon a certain cerebral disorder.

The solution of the above technical problem is achieved by providing the embodiments characterized in the claims.

In particular, the present invention is based on the fact that, beside promoting intact neurons, TGF-β is the major regulator for the execution of predamaged neurons upon a certain cerebral disorder. Accordingly, the present invention relates to the use of a compound capable of substantially inhibiting the biological activity of TGF-β on predamaged neurons, for treating cerebral disorders in mammals, preferably in man. The term "TGF-β" comprises the subspecies TGF-β1, TGF-β2, and TGF-β3.

In a preferred embodiment of the present invention the term "compound capable of substantially inhibiting the biological activity of TGF-β on predamaged neurons" refers to polyclonal or monoclonal antibodies directed to TGF-β, as TGFG-β inhibitors, or to antagonists directed to TGF-β such as compounds having the binding site of a TGF-β receptor, e.g. a TGF-β RII/Fc chimeric protein (TβR-II-Fc), or to proteinaceous or non-proteinaceous compounds which are capable of, e.g. chemically, altering TGF-β in the organism such that the altered TGF-β is rendered incapable of binding to TGF-β receptors, as well as to low molecular weight compounds such as chemical or non-proteinaceous compounds, as TGF-β-antagonists.

The cerebral disorder includes peripheral and/or CNS-disorders including cerebral and focal ischemiae such as apoplexy, and neurodegenerative disorders such as ALS. For example, adverse consequences of central nervous system injuries may

be caused by thrombus, embolus, systemic hypotension, hypertension, hypertensive cerebral vascular disease, rupture of an aneurysm, an angioma, blood dyscrasias, cardiac failure, cardic arrest, cardiogenic shock, septic shock, head trauma, spinal cord trauma, seizure, bleeding from a tumor, or other blood loss. The spinal cord, which is also a part of the central nervous system, is equally susceptible to ischemia resulting from diminished blood flow.

Where the ischemia is associated with a "stroke", it can be either global or focal ischemia, as defined below.

By "focal ischemia", as used herein in reference to the central nervous system, is meant the condition that results from the blockage of a single artery that supplies blood to the brain or spinal cord, resulting in the death of all cellular elements (pan-necrosis) in the territory supplied by that arterty.

By "global ischemia", as used herein in reference to the central nervous system, is meant the condition that results from a general diminution of blood flow to the entire brain, forebrain, or spinal cord, which causes the death of neurons in selectively vulnerable regions throughout these tissues. The pathology in each of these cases is quite different, as are the clinical correlates. Models of focal ischemia apply to patients with focal cerebral infarction, while models of global ischemia are analogous to cardiac arrest, and other causes of systemic hypotension.

A further aspect of the present invention relates to a pharmaceutical composition containing, in pharmaceutically effective amounts, the above defined compound, and a second compound for disintegrating blood clots, and optionally a pharmaceutically acceptable carrier and/or diluent. In a preferred embodiment of the present invention, the second compound is selected from the group consisting of urokinase, thrombin, and tPA (tissue plasminogen activator).

The treatment regimen is carried out, in terms of administration mode, timing of the administration, in dosage, so that the functional recovery of the patient from

the adverse consequence of the cerebral disorder is improved.

The administration of the compound or pharmaceutical composition according to the present invention can be carried out by any standard route and known rule of administration, including intravenously, orally, or intracerebrally. The dosage of such antibodies or antagonists according to the present invention lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized.

The formulation of the above defined compound or the pharmaceutical composition according to the present invention does not exhibit any specific restriction, and may be prepared e.g. in the form of tablets, suppositories, solutions, or retarded release-formulations. The antibodies or antagonists for example can be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, for example, in ampules or in multi-dose containers, with an added preservative.

The therapeutic antibodies or antagonists of the invention can also contain a carrier or excipient and/or diluent, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol.

It is well known in the medical arts that dosages for any one patient depend on many factors, including the general health, sex, weight, body surface area, and age of the patient, as well as the particular compound to be administered, in the time and route of administration, and other drugs being administered concurrently. Determining the most appropriate dosage and route of administration is well within the abilities of a skilled physician.

The figures show:

Fig. 1: Specifity of anti-TGF-β-antibody as well as morphology and neuron numbers of chick ganglia at E10, i.e. after the main period of ontogenetic ciliary neuron death. Embryos were treated daily from E6 to E9 with 10 μg of a monoclonal mouse anti-TGF-β1, -β2, -β3 antibody (anti-TGF-β; obtained from Genzyme) applied to the chorionic allantoic membrane, an identical dose of a mouse IgG with or without rhodamine conjugation, or 2 μg of a recombinant human TGF-β RII/Fc chimeric protein (TβR-II-Fc; obtained form R&D Systems). Ciliary ganglia were dissected at E10, fixed in Bouin's solution, and paraffin-embedded. (A) Digitalized photograph of a dot blot showing the specifity of the anti-TGF-β-antibody. (B) Photographs of sections (H.E. stain or fluorescence microscopy) through the largest circumference of ganglia from embryos with the indicated treatments. Bar = 100 μm. (C) Graphic representations of neuron counts in ganglia with the indicated treatments. Data are given as mean +/- SEM (n=5), p values derived from Student's t-test are $***p<0.001$ for increased neuron numbers as compared to untreated embryos.

Fig. 2: Neuron numbers and apoptosis in ciliary ganglia (CG), dorsal root ganglia (L3; DRG), and the lumbar motoneuron column of embryos treated with anti-TGF-β. (A) Graphic representation of neuron counts at E10 following daily treatments from E6 - E9. (B) Photographs of TUNEL labelings of CG, DRG, and lumbar motoneurons at E8 following treatment with anti-TGF-β-antibody at E6 and E7 (lower panel) as compared to untreated embryos (upper panel). Arrowheads point at TUNEL-positive nuclei. (C) Graphic representation of a quantitative analysis of TUNEL-positive neurons. Data in (A) and (C) are given as mean +/- SEM [n=5 in (A), n=3 in (C)], p values derived from Student's t-test are $**p<0.01$, $***p<0.001$ for increased (A) or decreased (C) neuron numbers as compared to untreated embryos.

Fig.3: Graphic representation of neuron counts showing phenotype rescue of CG and motoneurons in anti-TGF-ß-treated embryos by administration of TGF-ß. Treatment with TGF-ß3 (2μg) at E10 following administration of anti-TGF-ß from E6 to E9 (grey bars) produced neuron numbers at E14 (dashed bars) identical to control embryos (white bars). Continuing treatment with anti-TGF-ß from E10 to E 13 (black bars) fully maintained neuron numbers. Data are given as mean +/- SEM (n = 5), p values derived from Student's t-test are $**p<0.01$, $***p<0.001$ for increased neuron numbers as compared to controls, $+++p<0.001$ for decreased neuron numbers as compared to antibody-treated embryos.

Fig. 4: Qualitative microscopic and quantitative analyses showing that anti-TGF-ß rescues lumbar DRG and motoneurons following unilateral limb bud ablation. (A) Photographs of microscopic analyses of H.E. stained sections. Bar = 200 μm. (B) Graphic representation of neuron counts at E7 following the indicated treatments. Data are given as mean +/- SEM (n = 5), p values derived from Student's t-test are $***p<0.001$ for increased neuron numbers following limb bud ablation.

The present invention is illustrated by the following non-limiting example.

EXAMPLE

Neuron counting experiments

Chick embryos were treated daily with 50 μl (phosphate buffered saline, supplemented with 200 units/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml neomycin (each obtained from Gibco) containing either 10 μg of a pan anti-TGF-β (obtained from Genzyme) or 2 μg TβR-II-Fc (obtained from R&D Systems), or 10 μg mouse IgG (obtained from Sigma) by administration onto the chorio-

allantoic membrane through a window in the shell as described in Oppenheim et al., 1993. Embryos were killed on either E8, E10 or E14. The CG and the lumbar spinal cord with attached DRG were dissected, fixed in Bouin's solution and paraffin embedded. All tissues were sectioned at 8 to 10 μm and stained with H.E. Neurons were counted in every fifth or tenth section as described in Oppenheim et al., 1993.

TUNEL labeling

Sections were stained according to the manufacturer's instructions (Boehringer Mannheim) and counter-stained with H.E. TUNEL positive nuclei of large neurons were counted on every tenth section and expressed as ratio of total neuron of this particular neuron population.

Unilateral limb bud ablation

Ablations of hind limb buds of chick embryos were performed at E3. Embryos were treated daily (10 μg; E3 - E6) as indicated above with either anti-TGF-ß or IgG and processed at E7 for H.E. staining and neuron counting.

Anti-TGF-β-antibody neutralizes endogenous TGF-β during the main period of ontogenetic neuron cell death

In the developing nervous system of chick and mammals, TGF-ß2 and -ß3 as well as their receptors occur in many populations of postmitotic neurons, including CG, DRG, and motoneurons (Krieglstein et al., 1998). A monoclonal antibody recognizing all three isoforms of TGF-ß (anti-TGF-ß) was used to neutralize endogenous TGF-ß during the main period of ontogenetic cell death (E6 - E9) of CG and DRG as well as spinal motoneurons. Ten μg of this antibody neutralize 10 ng of each available recombinant TGF-ß including chicken TGF-ß3 (R&D Systems) to >98%, as assessed in a bioassay using mink lung epithelial cells (Abe et al., 1994; Krieglstein and Unsicker, 1995). The specifity of this monoclonal anti-TGF-ß antibody was ascertained by dot blot using 40 other

growth factors (Fig. 1A). Fig. 1B shows that daily treatments with anti-TGF-ß resulted in an increased size of CG at E10. A corresponding control IgG, which is rhodamine-conjugated, could be detected throughout the entire embryo. A TßR-II-Fc chimeric protein employed as an alternative tool to capture endogenous TGF-ß likewise increased the size of CG at E10 compared to untreated or IgG-treated embryos. Neuron counts (Fig. 1C) revealed that the increased size of CG is due to a significant increase in neuron numbers. The counted values of approximately 6,000 reflect neuron numbers prior to ontogenetic neuron death at E6 (Landmesser and Pilar, 1974) showing that elimination of TGF-ß signalling prevents the execution of ontogenetic neuron death in the chick CG.

Neutralizing TGF-ß interferes with ontogenetic cell death in sensory and motoneurons

Sensory and motoneurons were analyzed to investigate whether neutralizing TGF-ß also interfered with the development of other neuron populations during the period of ontogenetic neuron death. Neuron counts in the L3 DRG and in the lumbar motoneuron column revealed a significant increase in neuron numbers at E10 following daily antibody treatments from E6 to E9 (Fig. 2A). Numbers of motoneurons in antibody-treated embryos almost matched neuron numbers prior to the onset of ontogenetic neuron death (Hamburger et al., 1975). To determine whether the increase in numbers of neurons upon anti-TGF-ß-treatment resulted from reduced ontogenetic neuron death, TUNEL labelling was performed to stain for apoptotic cells. Counting the numbers of TUNEL-positive cells at E8, which is the peak of cell death for all three neuron populations investigated, revealed a significant reduction of the proportion of apoptotic cells (Fig. 2B, C). This shows that endogenous TGF-ß is essential for executing the cell death program in developing neurons.

Ontogenetic neuron death occurs after termination of antibody treatment and substitution of endogenous TGF-*β*

Furthermore, it was investigated whether ontogenetic neuron death would occur

at a later time point following termination of the antibody treatment and substitution of endogenous TGF-β. Fig. 3 shows that a single dose of TGF-β3 at E10 after the end of the antibody treatment (E6 -E9) resulted in a significant reduction of neuron numbers in the CG and motoneuron column. Numbers of neurons matched those of control embryos at the end of the main ontogenetic cell death period (E10) as well as embryos at E14. Therefore, TGF-β mediates death of selected neurons, especially those that are destined to die. In contrast, prolongation of the antibody treatment beyond the period of ontogenetic death stabilized neuron numbers at increased levels (Fig. 3).

Treatment of limb bud-ablated embryos with anti-TGF-β rescues both motoneurons and DRG neurons

Multiple evidence suggests that the extent of ontogenetic neuron death is crucially regulated by the target (Olek and Edwards, 1978; Pittman and Oppenheim, 1979; Pilar et al., 1980; Thoenen and Edgar, 1985), best exemplified by the classic experiments of V. Hamburger (Hollyday and Hamburger, 1976). Extirpation of the hind limb bud in the E3 chick embryo, i.e. prior to the arrival and synapse formation of motoneuron axons in the target (Dahm and Landmesser, 1988, 1991), causes death of almost all lumbar motoneurons and sensory neurons by E7 (Oppenheim et al., 1978) (Fig. 4A). Qualitative microscopic analysis (Fig. 4A) documents that treatment of limb bud-ablated embryos with anti-TGF-ß rescues both motoneurons as well as DRG neurons. Quantification reveals that the lumbar motoneuron population in limb-ablated embryos could surprisingly be rescued by anti-TGF-ß treatment to approximately half the half the size as compared to the non-operated control side (Fig. 4B).

Summary

The above results show that TGF-ß exerts a key role both in the regulation of ontogenetic neuron death of three major classes of peripheral and CNS neurons as well as in the regulation of cell death following target ablation. Parasympathetic CG, sensory DRG, and spinal lumbar motoneurons in chick embroys have

their main period of ontogenetic neuron death between E6 and E9, loose approximately 50% of the total cells generated, but require largely distinct and only partly overlapping target-derived trophic molecules to secure the survival of optimal cell numbers. CG neurons are maintained by CNTF/GPA, DRG neurons mainly by neurotrophins, and motoneurons by GDNF, HGF, neurotrophins, IGF-I and others (Nishi, 1994; Heller et al., 1995; Lewin and Barde, 1996; Oppenheim, 1996). In motoneurons, even a cocktail of numerous trophic molecules cannot fully maintain the population. The example of the present invention demonstrates that the elimination of endogenous TGF-β or TGF-β signaling, respectively, results in rescuing effects, which are distinct from neurotrophic factor treatments in terms of (i) the broad spectrum of responsive populations and (ii) magnitude of effect, which encompasses virtually all neurons of a given population. Therefore, the lack of TGF-β is able to rescue all neurons destined to die. The elimination of TGF-β is superior to any of known manipulations of the molecular cascade involved in neuron cell death in that neuron populations are fully maintained.

The example shows that ontogenetic neuron death of ciliary, dorsal root and spinal motoneurons is largely prevented and neuron losses following limb bud ablation are greatly reduced following neutralization of endogenous TGF-β by an anti-TGF-β antibody in chick embryos. Likewise, preventing TGF-β signaling by treatment with a TβR-II fusion protein during the period of ontogenetic cell death in the ciliary ganglion rescues all neurons, which normally die. TUNEL staining revealed decreased numbers of apoptotic cells. Application of exogenous TGF-β rescued the deprived phenotype. Thus, TGF-β in contrast to any single neurotrophic factor acting in the above systems plays a key role in regulating ontogenetic neuron death as well as cell death following neuronal target deprivation.

The above results demonstrate the pivotal role of TGF-β in regulating neuron survival and death. The data given above show that TGF-β is an essential trigger of neuronal death and thus, compounds according to the present invention which are capable of inhibiting the biological activity of TGF-β as e.g. TGF-β-specific antibodies are exceptionally good therapeutic tools that prevent TGF-β

signalling in the treatment of stroke, neurotrauma, and neurodegenerative diseases.

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"Use of TGF-inhibitors for treating cerebral disorders"

Claims

1. Use of a compound capable of substantially inhibiting the biological activity of TGF-β on predamaged neurons, for the preparation of a medicament for treating cerebral disorders.

2. Use according to claim 1, wherein said compound is an antibody or an antagonist directed to TGF-β.

3. Use according to claim 1 or 2, wherein said disorder is a peripheral and/or CNS-disorder.

4. Use according to claim 3, wherein said disorder is a cerebral ischemia or a neurodegenerative disorder.

5. A pharmaceutical composition containing, in pharmaceutically effective amounts, a compound capable of substantially inhibiting the biological activity of TGF-β on predamaged neurons, and a second compound for disintegrating blood clots, and optionally a pharmaceutically acceptable carrier and/or diluent.

6. The pharmaceutical composition according to claim 5, wherein said compound is an antibody directed to TGF-β or a compound having the binding site of a TGF-β receptor.

7. The pharmaceutical composition according to claim 5 or 6, wherein said second compound is selected from the group consisting of urokinase, thrombin, and tissue plasminogen activator.

The figures show:

Fig. 1: Specifity of anti-TGF-β-antibody as well as morphology and neuron numbers of chick ganglia at E10, i.e. after the main period of ontogenetic ciliary neuron death. Embryos were treated daily from E6 to E9 with 10 μg of a monoclonal mouse anti-TGF-β1, -β2, -β3 antibody (anti-TGF-β; obtained from Genzyme) applied to the chorionic allantoic membrane, an identical dose of a mouse IgG with or without rhodamine conjugation, or 2 μg of a recombinant human TGF-β RII/Fc chimeric protein (TβR-II-Fc; obtained form R&D Systems). Ciliary ganglia were dissected at E10, fixed in Bouin's solution, and paraffin-embedded. (A) Digitalized photograph of a dot blot showing the specifity of the anti-TGF-β-antibody. (B) Photographs of sections (H.E. stain or fluorescence microscopy) through the largest circumference of ganglia from embryos with the indicated treatments. Bar = 100 μm. (C) Graphic representations of neuron counts in ganglia with the indicated treatments. Data are given as mean +/- SEM (n=5), p values derived from Student's t-test are $***p<0.001$ for increased neuron numbers as compared to untreated embryos.

Fig. 2: Neuron numbers and apoptosis in ciliary ganglia (CG), dorsal root ganglia (L3; DRG), and the lumbar motoneuron column of embryos treated with anti-TGF-β. (A) Graphic representation of neuron counts at E10 following daily treatments from E6 - E9. (B) Photographs of TUNEL labelings of CG, DRG, and lumbar motoneurons at E8 following treatment with anti-TGF-β-antibody at E6 and E7 (lower panel) as compared to untreated embryos (upper panel). Arrowheads point at TUNEL-positive nuclei. (C) Graphic representation of a quantitative analysis of TUNEL-positive neurons. Data in (A) and (C) are given as mean +/- SEM [n=5 in (A), n=3 in (C)], p values derived from Student's t-test are $**p<0.01$, $***p<0.001$ for increased (A) or decreased (C) neuron numbers as compared to untreated embryos.

Fig.3: Graphic representation of neuron counts showing phenotype rescue of CG and motoneurons in anti-TGF-ß-treated embryos by administration of TGF-ß. Treatment with TGF-ß3 (2μg) at E10 following administration of anti-TGF-ß from E6 to E9 (grey bars) produced neuron numbers at E14 (dashed bars) identical to control embryos (white bars). Continuing treatment with anti-TGF-ß from E10 to E 13 (black bars) fully maintained neuron numbers. Data are given as mean +/- SEM (n=5), p values derived from Student's t-test are $**p<0.01$, $***p<0.001$ for increased neuron numbers as compared to controls, $+++p<0.001$ for decreased neuron numbers as compared to antibody-treated embryos.

Fig. 4: Qualitative microscopic and quantitative analyses showing that anti-TGF-ß rescues lumbar DRG and motoneurons following unilateral limb bud ablation. (A) Photographs of microscopic analyses of H.E. stained sections. Bar = 200 μm. (B) Graphic representation of neuron counts at E7 following the indicated treatments. Data are given as mean +/- SEM (n=5), p values derived from Student's t-test are $***p<0.001$ for increased neuron numbers following limb bud ablation.

The present invention is illustrated by the following non-limiting example.

EXAMPLE

Neuron counting experiments

Chick embryos were treated daily with 50 μl (phosphate buffered saline, supplemented with 200 units/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml neomycin (each obtained from Gibco) containing either 10 μg of a pan anti-TGF-β (obtained from Genzyme) or 2 μg TβR-II-Fc (obtained from R&D Systems), or 10 μg mouse IgG (obtained from Sigma) by administration onto the chorio-

allantoic membrane through a window in the shell as described in Oppenheim et al., 1993. Embryos were killed on either E8, E10 or E14. The CG and the lumbar spinal cord with attached DRG were dissected, fixed in Bouin's solution and paraffin embedded. All tissues were sectioned at 8 to 10 μm and stained with H.E. Neurons were counted in every fifth or tenth section as described in Oppenheim et al., 1993.

TUNEL labeling

Sections were stained according to the manufacturer's instructions (Boehringer Mannheim) and counter-stained with H.E. TUNEL positive nuclei of large neurons were counted on every tenth section and expressed as ratio of total neuron of this particular neuron population.

Unilateral limb bud ablation

Ablations of hind limb buds of chick embryos were performed at E3. Embryos were treated daily (10 μg; E3 - E6) as indicated above with either anti-TGF-ß or IgG and processed at E7 for H.E. staining and neuron counting.

Anti-TGF-β-antibody neutralizes endogenous TGF-β during the main period of ontogenetic neuron cell death

In the developing nervous system of chick and mammals, TGF-ß2 and -ß3 as well as their receptors occur in many populations of postmitotic neurons, including CG, DRG, and motoneurons (Krieglstein et al., 1998). A monoclonal antibody recognizing all three isoforms of TGF-ß (anti-TGF-ß) was used to neutralize endogenous TGF-ß during the main period of ontogenetic cell death (E6 - E9) of CG and DRG as well as spinal motoneurons. Ten μg of this antibody neutralize 10 ng of each available recombinant TGF-ß including chicken TGF-ß3 (R&D Systems) to >98%, as assessed in a bioassay using mink lung epithelial cells (Abe et al., 1994; Krieglstein and Unsicker, 1995). The specifity of this monoclonal anti-TGF-ß antibody was ascertained by dot blot using 40 other

growth factors (Fig. 1A). Fig. 1B shows that daily treatments with anti-TGF-ß resulted in an increased size of CG at E10. A corresponding control IgG, which is rhodamine-conjugated, could be detected throughout the entire embryo. A TßR-II-Fc chimeric protein employed as an alternative tool to capture endogenous TGF-ß likewise increased the size of CG at E10 compared to untreated or IgG-treated embryos. Neuron counts (Fig. 1C) revealed that the increased size of CG is due to a significant increase in neuron numbers. The counted values of approximately 6,000 reflect neuron numbers prior to ontogenetic neuron death at E6 (Landmesser and Pilar, 1974) showing that elimination of TGF-ß signalling prevents the execution of ontogenetic neuron death in the chick CG.

Neutralizing TGF-ß interferes with ontogenetic cell death in sensory and motoneurons

Sensory and motoneurons were analyzed to investigate whether neutralizing TGF-ß also interfered with the development of other neuron populations during the period of ontogenetic neuron death. Neuron counts in the L3 DRG and in the lumbar motoneuron column revealed a significant increase in neuron numbers at E10 following daily antibody treatments from E6 to E9 (Fig. 2A). Numbers of motoneurons in antibody-treated embryos almost matched neuron numbers prior to the onset of ontogenetic neuron death (Hamburger et al., 1975). To determine whether the increase in numbers of neurons upon anti-TGF-ß-treatment resulted from reduced ontogenetic neuron death, TUNEL labelling was performed to stain for apoptotic cells. Counting the numbers of TUNEL-positive cells at E8, which is the peak of cell death for all three neuron populations investigated, revealed a significant reduction of the proportion of apoptotic cells (Fig. 2B, C). This shows that endogenous TGF-ß is essential for executing the cell death program in developing neurons.

Ontogenetic neuron death occurs after termination of antibody treatment and substitution of endogenous TGF-β

Furthermore, it was investigated whether ontogenetic neuron death would occur

at a later time point following termination of the antibody treatment and substitution of endogenous TGF-β. Fig. 3 shows that a single dose of TGF-β3 at E10 after the end of the antibody treatment (E6 -E9) resulted in a significant reduction of neuron numbers in the CG and motoneuron column. Numbers of neurons matched those of control embryos at the end of the main ontogenetic cell death period (E10) as well as embryos at E14. Therefore, TGF-β mediates death of selected neurons, especially those that are destined to die. In contrast, prolongation of the antibody treatment beyond the period of ontogenetic death stabilized neuron numbers at increased levels (Fig. 3).

Treatment of limb bud-ablated embryos with anti-TGF-β rescues both motoneurons and DRG neurons

Multiple evidence suggests that the extent of ontogenetic neuron death is crucially regulated by the target (Olek and Edwards, 1978; Pittman and Oppenheim, 1979; Pilar et al., 1980; Thoenen and Edgar, 1985), best exemplified by the classic experiments of V. Hamburger (Hollyday and Hamburger, 1976). Extirpation of the hind limb bud in the E3 chick embryo, i.e. prior to the arrival and synapse formation of motoneuron axons in the target (Dahm and Landmesser, 1988, 1991), causes death of almost all lumbar motoneurons and sensory neurons by E7 (Oppenheim et al., 1978) (Fig. 4A). Qualitative microscopic analysis (Fig. 4A) documents that treatment of limb bud-ablated embryos with anti-TGF-ß rescues both motoneurons as well as DRG neurons. Quantification reveals that the lumbar motoneuron population in limb-ablated embryos could surprisingly be rescued by anti-TGF-ß treatment to approximately half the half the size as compared to the non-operated control side (Fig. 4B).

Summary

The above results show that TGF-ß exerts a key role both in the regulation of ontogenetic neuron death of three major classes of peripheral and CNS neurons as well as in the regulation of cell death following target ablation. Parasympathetic CG, sensory DRG, and spinal lumbar motoneurons in chick embroys have

their main period of ontogenetic neuron death between E6 and E9, loose approximately 50% of the total cells generated, but require largely distinct and only partly overlapping target-derived trophic molecules to secure the survival of optimal cell numbers. CG neurons are maintained by CNTF/GPA, DRG neurons mainly by neurotrophins, and motoneurons by GDNF, HGF, neurotrophins, IGF-I and others (Nishi, 1994; Heller et al., 1995; Lewin and Barde, 1996; Oppenheim, 1996). In motoneurons, even a cocktail of numerous trophic molecules cannot fully maintain the population. The example of the present invention demonstrates that the elimination of endogenous TGF-β or TGF-β signaling, respectively, results in rescuing effects, which are distinct from neurotrophic factor treatments in terms of (i) the broad spectrum of responsive populations and (ii) magnitude of effect, which encompasses virtually all neurons of a given population. Therefore, the lack of TGF-β is able to rescue all neurons destined to die. The elimination of TGF-β is superior to any of known manipulations of the molecular cascade involved in neuron cell death in that neuron populations are fully maintained.

The example shows that ontogenetic neuron death of ciliary, dorsal root and spinal motoneurons is largely prevented and neuron losses following limb bud ablation are greatly reduced following neutralization of endogenous TGF-β by an anti-TGF-β antibody in chick embryos. Likewise, preventing TGF-β signaling by treatment with a TβR-II fusion protein during the period of ontogenetic cell death in the ciliary ganglion rescues all neurons, which normally die. TUNEL staining revealed decreased numbers of apoptotic cells. Application of exogenous TGF-β rescued the deprived phenotype. Thus, TGF-β in contrast to any single neurotrophic factor acting in the above systems plays a key role in regulating ontogenetic neuron death as well as cell death following neuronal target deprivation.

The above results demonstrate the pivotal role of TGF-β in regulating neuron survival and death. The data given above show that TGF-β is an essential trigger of neuronal death and thus, compounds according to the present invention which are capable of inhibiting the biological activity of TGF-β as e.g. TGF-β-specific antibodies are exceptionally good therapeutic tools that prevent TGF-β

signalling in the treatment of stroke, neurotrauma, and neurodegenerative diseases.

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"Use of TGF-inhibitors for treating cerebral disorders"

Claims

1. Use of a compound capable of substantially inhibiting the biological activity of TGF-β on predamaged neurons, for the preparation of a medicament for treating cerebral disorders.

2. Use according to claim 1, wherein said compound is an antibody or an antagonist directed to TGF-β.

3. Use according to claim 1 or 2, wherein said disorder is a peripheral and/or CNS-disorder.

4. Use according to claim 3, wherein said disorder is a cerebral ischemia or a neurodegenerative disorder.

5. A pharmaceutical composition containing, in pharmaceutically effective amounts, a compound capable of substantially inhibiting the biological activity of TGF-β on predamaged neurons, and a second compound for disintegrating blood clots, and optionally a pharmaceutically acceptable carrier and/or diluent.

6. The pharmaceutical composition according to claim 5, wherein said compound is an antibody directed to TGF-β or a compound having the binding site of a TGF-β receptor.

7. The pharmaceutical composition according to claim 5 or 6, wherein said second compound is selected from the group consisting of urokinase, thrombin, and tissue plasminogen activator.